Dietary supplementation with anthocyanin attenuates lipopolysaccharide-induced intestinal damage through antioxidant effects in yellow-feathered broiler chicks

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ABSTRACT This study investigated the protective effects of anthocyanin (**AC**) supplementation on lipopolysaccharide (**LPS**)-challenged yellow-feathered broiler chicks. A total of 480 1-d female broiler chicks were randomly assigned to 4 treatment groups: basal diet (**CON**), basal diet + LPS-challenge (**LPS**), supplementation with 100 or 400 mg/kg AC + LPS-challenge (**AC100, AC400**). On d 17 and d 19, birds in LPS, AC100 and AC400 received an intramuscular dose of LPS, while birds in CON received saline. The result showed that (1) LPS injection significantly decreased ($P < 0.05$) body weight on d 21 and average daily gain of broiler chicks from 1 to 21 days of age, and supplementation with 100 mg/kg AC increased ($P < 0.05$) those of LPS-challenged broilers. (2) There were no differences among the treatments ($P > 0.05$) in relative weights of immune organs. (3) Supplementation with AC (AC100 and AC400) increased ($P < 0.05$) the jejunal villus height and villus height/crypt depth ratio (AC100) of LPS-challenged birds. Challenge with LPS decreased the relative expression of *OCLN* (*Occludin*), *ZO-1, JAM2*, and *MUC2* in jejunal mucosa of broilers, and supplementation with AC offset the relative expression of *ZO-1, JAM2* (AC100 and AC400), and *OCLN* (AC400) in LPS-injected broilers. (4) LPS-induced increase in the malondialdehyde (**MDA**) concentration and decreases in activity of total superoxide dismutase (**T-SOD**), and expression of *SOD1, CAT* and *GPX* in jejunal mucosa, were attenuated by dietary AC supplementation. In conclusion, in yellow-feathered broiler chicks, dietary supplementation with AC alleviated LPS-induced declined growth performance and mucosal damage of the intestine through antioxidant effects.

Key words: anthocyanin, broiler, lipopolysaccharide, intestinal damage, antioxidant effect

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INTRODUCTION

Intestinal mucosa is necessary for maintaining intestinal homeostasis and preventing pathogen invasion of broilers (Jiang et al., 2019). In the poultry industry, varieties of stress, including mildewed feed, pathogenic invasion, oxidative stress, and immunological stress, injure the intestinal mucosa, and lead to the reduced performance and unhealthy status of broilers, resulting in great economic losses. Oxidative stress with excess generation of reactive oxygen species (**ROS**), and toxic free radicals, leads to the imbalance of redox homeostasis (Zheng et al., 2016). Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, induced ROS production and damage to lipids, producing malondialdehyde (**MDA**), a biomarker of lipid degradation, resulting in serious oxidative stress (Brandes et al., 1999; Sun et al., 2020). LPS challenge impairs the integrity and oxidative status of the intestine, and causes loss of growth performance of broilers; it is widely used as a model of oxidative stress in broiler chicks (Jiang et al., 2019; Sun et al., 2020; Zheng et al., 2020).

Nutrition-based strategies such as effective feed additives are urgently needed to address problems related to oxidative stress in young poultry. Previous work has shown that oxidative stress induced by LPS can be alleviated by dietary supplementation with plant extracts showing antioxidant activity, for example, glycyrrhiza polysaccharide (Zhang et al., 2021a,b), leonurine hydrochloride (Yang et al., 2019), bisdemethoxycurcumin (Zhang et al., 2021a,b), and carvacrol essential oils (Liu et al., 2019).

Anthocyanin (**AC**) is ubiquitously found in plants such as plums, blackberries, cherries, raspberries, grapes,

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and purple cabbage (Gomes et al., 2019), and exerts a series of pharmacological effects, such as antioxidant, anti-inflammatory, antibacterial, and anti-mutagenic actions (Castañeda-Ovando et al., 2009; Lin et al., 2017). The protective effects of AC against oxidative stress were demonstrated with in vitro models, such as H_2O_2-induced HepG2 cells (Chen et al., 2019), UVA-induced immortalized fibroblasts (Petruk et al., 2017) and UVB-induced HaCaT cells (He et al., 2017). Little is presently known of the antioxidant effects of dietary AC supplementation in poultry and livestock. This study was carried out to investigate the potential protective effects of supplementation with AC on attenuating intestinal damage of yellow-feathered broiler chicks caused by LPS, thus, to provide a basis for application of new feed additive products in broiler production.

MATERIALS AND METHODS

Experimental Design

The experiment was approved by the Animal Care Committee of the Institute of Animal Science, Guangdong Academy of Agriculture Science, Guangzhou, P. R. China, with the approval number of GAASIAS-2019-011.

A total of 480 1-d female, yellow-feathered broilers (fast-growing strain, 42.2 ± 0.30 g) were randomly assigned to 4 treatments (Table 1), each with 6 replicates of 20 broilers. Birds in the control group (**CON**) and LPS-challenged treatment (**LPS**) were fed a basal diet, and birds in the other 2 treatments received the basal diet with 100 or 400 mg/kg added AC (**AC100, AC400**). On days 17 and 19 of the trial, birds in LPS, AC100 and AC400 treatments received an intramuscular administration of LPS (500 μg/kg body weight), while birds in CON received an equal amount of PBS.

Anthocyanin (61% purity, an ethanolic extract of bilberry) was obtained from Tianjin Jianfeng Natural Product R&D Co., Ltd (Tianjin, China). LPS from *Escherichia coli* serotype O55:B5 was purchased (L2880, Sigma-Aldrich Chemical Co. Ltd, St. Louis, MO).

Diets and Chicken Husbandry

The diets were formulated as recommended by Chinese Nutrient Requirements of Yellow Chickens (Ministry of Agriculture and Rural Affairs, 2020). Anthocyanin was added to AC100 and AC400 diets in the premixes. Details of ingredient composition and calculated nutrient contents of the basal diets were given in Table 2.

Table 1. Experimental design.

Treatment	CON	LPS	AC100	AC400
Dietary supplementation with anthocyanin, mg/kg	-	-	100	400
Injection of LPS	-	+	+	+

AC100, basal diet supplemented with 100 mg/kg anthocyanin + LPS challenge; AC400, basal diet supplemented with 400 mg/kg anthocyanin + LPS challenge; CON, basal diet + saline injection; LPS, basal diet + LPS challenge.

Table 2. Composition and nutrient levels of the basal diet.

Ingredients	%	Nutrient levels[2]	%
Corn	57.60	ME/(MJ/kg)	12.12
Soybean meal	34.20	CP	20.36
Soybean oil	2.10	Ca	1.02
Limestone	1.20	P	0.70
$CaHPO_4$	1.90	Nonphytate phosphorus P	0.46
NaCl	0.30	Lys	1.16
DL-Met (99%)	0.15	Met	0.46
Premix[1]	1.00		
Rice bran	1.55		
Total	100.00		

[1]Premix provided the following per kilogram of the diet during 1 to 21 d of age: VA15 000 IU, VD_3 3 300 IU, VE 20 IU, VK_3 4.0 mg, VB_1 1.8 mg, VB_2 9.0 mg, VB_6 3.5 mg, VB_{12} 0.01 mg, choline chloride 500 mg, niacin 60 mg, pantothenic acid 16 mg, folic acid 0.55 mg, biotin 0.15 mg, Fe 80 mg, Cu 8 mg, Mn 80 mg, Zn 60 mg, I 0.35 mg, Se 0.30 mg.

[2]CP, Ca and P were measured values, while the others were calculated.

The 21-day experiment was carried out in the testing farm of Institute of Animal Science, Guangdong Academy of Agricultural Sciences. Birds were raised in floor pens with wood shavings litter, with the stocking density of 0.20 m^2/bird. Water and diets were provided ad libitum throughout. The room temperature was kept at 32 to 34°C for the first 3 d and reduced by 2°C per week. The light cycle with incandescent bulbs was 23L:1D for the first 3 d, 21L:3D from d 4 to d 10, and 18L:6D from d 11 (Wang et al., 2020).

Measurement of Growth Performance

Birds were weighed at d 1 and d 21 on a per pen basis. The final body weight, average daily gain, average daily feed intake and feed to gain ratio were calculated, as described (Wang et al., 2020).

Sample Collection

At the end of the experiment (d 21), after 12 h of feed-withdrawal, 2 birds close to average body weight per replicate were weighed, and then electrically stunned and exsanguinated. Blood samples were collected from the jugular vein into 5 mL heparinized tubes, and plasma was then obtained after centrifuging at 1,000 × *g* for 15 min at 4°C. After opening lengthwise, mid-jejunal segments were rinsed with sterile saline and portions were fixed by immersion in 4% paraformaldehyde. The mucosa was collected by gentle scraping of additional portions of the jejunum. Portions of liver and jejunal mucosal samples were snap-frozen in liquid N_2 and kept at −80°C.

Morphological Observation of Jejunum

Five μm paraffin-embedded sections of jejunum were cut, mounted then dewaxed in xylene, rehydrated with an ethanolic series and routinely stained with H&E, to

observe the morphology of villi. The height of villi, and depth of adjacent crypts were examined microscopically with a Panoramic Scanner (P-MIDI P250, 3D Histech, HUN), and the villus height to crypt depth was calculated.

Calculation of Relative Weights of Immune Organs

For the above broilers, the liver, spleen, thymus and bursa of Fabricius were dissected, blotted and weighed. The relative weights (weight of organ/body weight × 100%) were calculated.

Determination of Immunoglobulin Contents

The contents of IgY, IgM, and sIgA in plasma and jejunal mucosa were determined with ELISA kits (MM050501, MM091201 and MM049301, Jiangsu Meimian industrial Co., Ltd, Zhangjiagang, China) and a spectrophotometer (Spectra Max M-5, Molecular Devices, San Jose, CA)

Determination of Antioxidative Variables

Samples of liver and jejunal mucosa were homogenized with 10 volumes of ice-cold physiologic saline and centrifuged at 2,000 × g for 10 min to obtain clarified extracts. Commercial kits (A003-1-2, A001-1-2, A005-1-2 and A007-1-1, Nanjing Jiancheng Institute of Bioengineering, Nanjing, China) and the spectrophotometer mentioned above were used to determine the content of MDA, the activities of total superoxide dismutase (**T-SOD**) and glutathione peroxidase (**GSH-Px**) in plasma, and extracts of liver and jejunal mucosa, along with catalase (**CAT**) in liver and jejunal mucosa.

Quantitative PCR

Total RNA was extracted from jejunal mucosa using RNAiso plus (9109, TAKARA, Tokyo, JP) and reverse-transcribed with the PrimeScript II 1st Strand cDNA Synthesis Kit (6210A, TAKARA). Real-time PCR was performed with SYBR PremixExTaq II (RR820A, TAKARA) and the real-time PCR system (ABI 7500, Applied Biosystems, Carlsbad, CA). The primers used were shown in Table 3. Results were normalized to the abundance of β-actin transcripts and relative quantification was calculated using the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

Effects of treatment were analyzed by the one-way analysis of variance (**ANOVA**) procedure in SPSS 17.0 (SPSS Inc., Chicago, IL). When treatment effects were significant ($P < 0.05$), means were separated by Duncan's multiple range test. Tabulated results were shown as means with standard error of mean (**SEM**).

Table 3. Primer sequences for real-time PCR.

Gene	GenBank ID	Primers sequence (5' to 3')	Tm, °C
β-actin	NM_001001611.2	F: GAGAAATTGTGCGTGACATCA R: CCTGAACCTCTCATTGCCA	56
CLDN	NM_001013611.2	F: GAGGATGACCAGGTCAAGAAG R: TGCCCAGCCAATGAAGAG	56
OCLN	XM_015278981.1	F: TCATCCTGCTCTGCCTCATCT R: CATCCGCCACGTTCTTCAC	56
ZO-1	XM_015278981.1	F: CCAAAGACAGCAGGAGGAGA R: TGGCTAGTTTCTCTCGTGCA	56
JAM2	NM_001083366.1	F: AGACAGGAACAGGCAGTGCT R: TCCAATCCCATTTGAGGCTA	60
MUC2	NM_001318434.1	F: CATTCAACGAGGAGAGCTGC R: TTCCTTGCAGCAGGAACAAC	56
Nrf-2	MN416129.1	F: ATCACCTCTTCTGCACCGAA R: GCTTTCTCCCGCTCTTTCTG	60
SOD1	NM_205064.1	F: GGTGCTCACTTTAATCCTG R: CTACTTCTGCCACTCCTCC	60
CAT	NM_001031215.2	F: AGACATCTTCGCTGTGGTGA R: CGAGGATGTTGATGCAGGTG	60
GPx-1	HM590226.1	F: ACGGCGCATCTTCCAAAG R: TGTTCCCCCAACCATTTCTC	60
NOX2	BR000270.1	F: TTGTCAAATGCCCAGCAGTG R: TCCACAGGCATTGAACAAGC	59

CAT, catalase; *CLDN1*, claudin 1; *GPx-1*, cytosolic glutathione peroxidase-1; *JAM*, junctional adhesion protein; MUC2 = mucin 2; *NOX* = nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; *Nrf-2* = nuclear factor, erythroid 2 like 2; *OCLN*, occludin; *SOD1* = superoxide dismutase 1; *ZO1*, tight junction protein 1.

RESULTS

Effects of AC Supplementation on Growth Performance of Broiler Chicks Challenged With LPS

The effects of AC supplementation on growth performance from 1 to 21 d of age of LPS-challenged broiler chicks were shown in Table 4. Compared with control birds, LPS injection significantly decreased ($P < 0.05$) the final body weight and average daily gain. For challenged birds, supplementation with 100 mg/kg AC restored ($P < 0.05$) the body weight and average daily gain.

Effects of AC Supplementation on Relative Weights of Immune Organs of Broiler Chicks Challenged With LPS

As shown in Table 5, there were no differences among the treatments ($P > 0.05$) in relative weights of liver, spleen, thymus or bursa of Fabricius.

Effects of AC Supplementation on Immunoglobulin Contents of Broiler Chicks Challenged With LPS

As shown in Table 6, there were no differences ($P > 0.05$) among treatments in plasma concentrations of immunoglobulins of birds. Compared with control birds, challenge with LPS significantly decreased the IgY content in jejunal mucosa ($P < 0.05$) but this was

Table 4. Effects of anthocyanin supplementation on growth performance of LPS-challenged broiler chicks from 1 to 21 days of age.[1]

Variables	CON	LPS	AC100	AC400	SEM	*P* value
Final body weight, g	360.83[a]	345.57[b]	359.20[a]	351.40[ab]	4.50	0.028
Average daily gain, g	15.18[a]	14.46[b]	15.10[ab]	14.73[b]	0.21	0.046
Average daily feed intake, g	33.02	31.57	31.92	31.95	0.53	0.252
Feed to gain ratio	2.18	2.16	2.13	2.19	0.03	0.433
Survival rate, %	100.00	100.00	99.17	100.00	0.42	0.413

[1]Values are means of six replicate pens (20 birds per pen).
[ab]Within a row, values with different letter superscripts mean significant difference ($P < 0.05$).
AC100, basal diet supplemented with 100 mg/kg anthocyanin + LPS challenge; AC400, basal diet supplemented with 400 mg/kg anthocyanin + LPS challenge CON, basal diet + saline injection; LPS, basal diet + LPS challenge;.

Table 5. Effects of anthocyanin supplementation on relative weights of immune organs of 21-d broiler chicks challenged with LPS.[1]

Organs	CON	LPS	AC100	AC400	SEM	*P* value
Liver/Body weight	2.81	2.76	2.79	0.68	0.06	0.304
Spleen/Body weight	0.18	0.20	0.18	0.19	0.01	0.746
Thymus/Body weight	0.38	0.40	0.38	0.43	0.03	0.225
Bursa of Fabricius/ Body weight	0.29	0.30	0.26	0.26	0.02	0.089

[1]Values are means of six replicates (2 birds per replicate).
AC100, basal diet supplemented with 100 mg/kg anthocyanin + LPS challenge; AC400, basal diet supplemented with 400 mg/kg anthocyanin + LPS challenge; CON, basal diet + saline injection; LPS, basal diet + LPS challenge.

Table 6. Effects of anthocyanin supplementation on immunoglobulin contents of 21-d broiler chicks challenged with LPS.[1]

Variables	CON	LPS	AC100	AC400	SEM	*P* value
Plasma						
IgA, μg /mL	6.95	6.40	6.57	6.74	.022	0.452
IgY, μg /mL	99.36	97.07	99.57	99.26	2.17	0.885
IgM, μg /mL	4.47	4.02	4.20	4.53	0.27	0.615
Jejunal mucosa						
IgA, μg /mg pro	9.31	9.43	9.62	9.58	0.17	0.675
IgY, μg / mg pro	69.10[a]	60.05[b]	62.41[a,b]	69.14[a]	2.41	0.029
IgM, μg / mg pro	8.69	7.57	7.55	9.42	0.56	0.101

[1]Values are means of six replicates (2 birds per replicate).
[ab]Within a row, values with different letter superscripts mean significant difference ($P < 0.05$).
AC100, basal diet supplemented with 100 mg/kg anthocyanin + LPS challenge; AC400, basal diet supplemented with 400 mg/kg anthocyanin + LPS challenge; CON, basal diet + saline injection; LPS, basal diet + LPS challenge.

completely offset by supplementation with 400 mg/kg AC (AC400).

Effects of AC Supplementation on Jejunal Morphology of Broiler Chicks Challenged With LPS

The jejunal morphology of broiler chicks was presented in Figure 1. Compared with controls, birds challenged with LPS showed significantly decreased ($P < 0.05$) villus height and villus height/crypt depth ratio (Figure 1**A**). For challenged birds, supplementation with 100 mg/kg AC increased the villus height and villus height/crypt depth ratio ($P < 0.05$), while supplementation with 400 mg/kg AC increased the villus height ($P < 0.05$). H&E stained jejunal section (Figure 1**B**) showed that the villi of CON birds were intact, whereas the those from birds treated with LPS were sparse and deranged. In challenged birds supplemented with AC, the derangement was lessened.

Effects of AC Supplementation on Antioxidative Variables of Broiler Chicks Challenged With LPS

As presented in Table 7, compared to control birds, challenge with LPS decreased the activity of T-SOD ($P < 0.05$) and increased the content of MDA ($P < 0.05$) in jejunal mucosa. Compared to LPS-treated birds, 100 mg/kg AC supplementation increased the activities of T-SOD and GSH-Px both in jejunal mucosa and liver ($P < 0.05$); supplementation with 400 mg/kg AC increased the activity of T-SOD both in plasma and liver ($P < 0.05$), and decreased the MDA content in liver ($P < 0.05$).

Effects of AC Supplementation on Gene Expression in Jejunum of Broiler Chicks Challenged With LPS

The effects of AC supplementation on relative transcript abundance in jejunal mucosa of LPS-challenged broiler chicks were shown in Figure 2. Compared with control birds, LPS challenge significantly decreased ($P < 0.05$) the relative expression of *OCLN, ZO-1, JAM2, MUC2, Nrf2, SOD1, CAT*, and *GPx-1* in jejunal mucosa. Compared with birds challenged with LPS, supplementation with 100 mg/kg AC increased the relative expression of *ZO-1* and *JAM2* ($P < 0.05$); supplementation with 400 mg/kg AC increased the relative expression of *ZO-1, JAM2* and *OCLN* in jejunal mucosa ($P < 0.05$).

DISCUSSION

Effects of AC Supplementation on Growth Performance of LPS-Challenged Broiler Chicks

In the poultry industry, a variety of stresses, including mildew-contaminated feeds, pathogenic invasion, oxidative stress and immunological stress, led to the reduced production performance of broilers. Lipopolysaccharide, an endotoxin derived from Gram-negative bacteria, is often used to establish oxidative stress models in animals (Zhang et al., 2021a,b). Previous researches showed that challenge with LPS decreased the weight gain of Ross

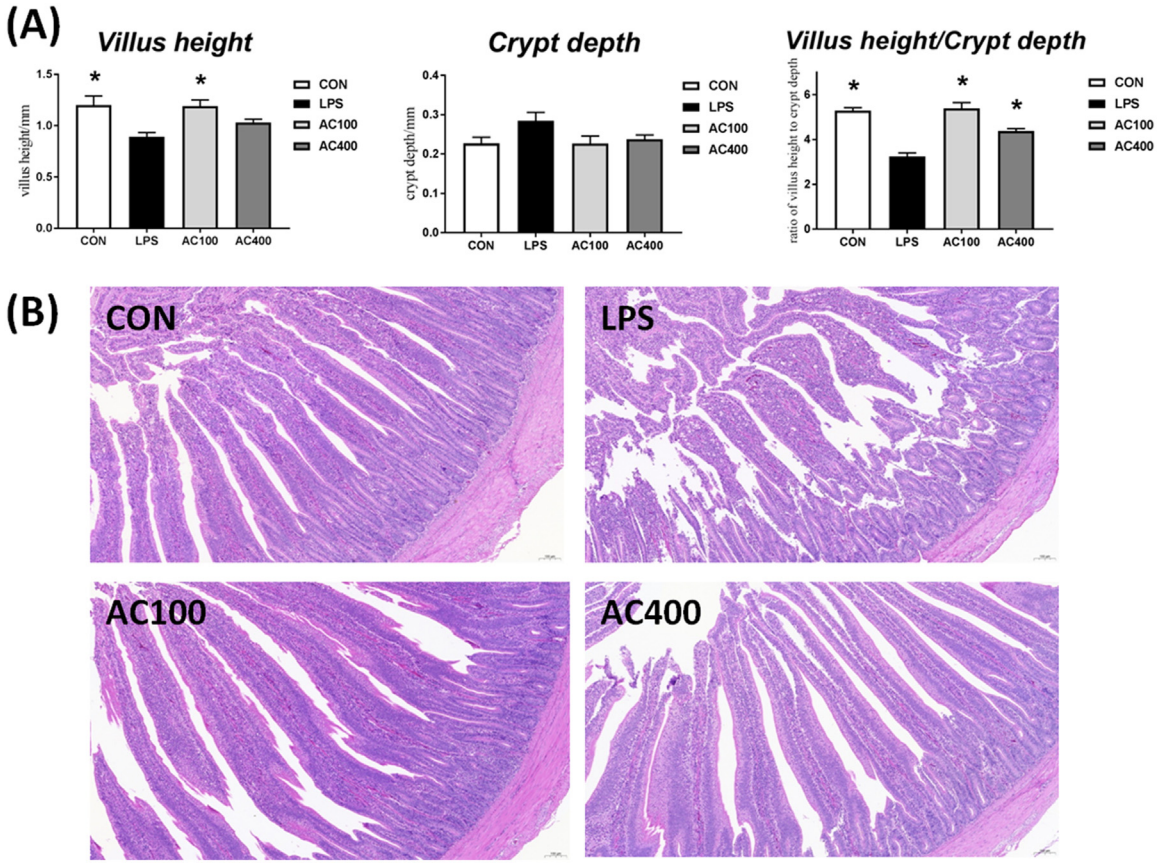

Figure 1. The effects of anthocyanin supplementation on jejunal morphology of LPS-challenged broiler chicks. (**A**) Villus height, crypt depth and the ratio between them among the treatments. (**B**) Representative images of H&E stained jejunal tissue. CON = basal diet + saline injection; LPS = basal diet + LPS challenge; AC100 = basal diet supplemented with 100 mg/kg anthocyanin + LPS challenge; AC400 = basal diet supplemented with 400 mg/kg anthocyanin + LPS challenge. Values are means ± SEM. *$P < 0.05$ compared with the LPS treatment.

broilers (Gadde et al., 2017; Yang et al., 2019) and Arbor Acre broiler chickens (Zheng et al., 2016; Zhang et al., 2021a,b). Similarly, in the current research, LPS injection here significantly decreased final body weight and average daily gain of yellow-feathered broiler chicks from 1 to 21 d of age.

For LPS-challenged birds, dietary supplementation with 100 mg/kg AC increased the body weight and average daily gain. There was little currently known about the application of AC to livestock and poultry. Based on another study from this laboratory (Wang et al., 2021), supplementation with AC had no influence on the growth performance of yellow-feathered broilers. Similarly, AC-rich roselle did not improve the growth of Ross 308 broilers (Amer et al., 2022). These together with the current study indicated that dietary

Table 7. Effects of supplementation with bilberry extract on antioxidant variables of 21-d broiler chicks challenged with LPS.[1]

Variables	CON	LPS	AC100	AC400	SEM	*P* value
Plasma						
GSH-PX, U/ml	1952.73	1831.36	1969.09	1960.91	93.57	0.709
T-SOD, U/mL	76.25^{ab}	69.73^{b}	78.68^{ab}	85.66^{a}	4.50	0.048
MDA, nmol/mL	1.40	1.63	1.52	1.38	0.16	0.697
Jejunal mucosa						
GSH-PX, U/mg pro	126.89^{b}	135.70^{b}	190.85^{a}	150.96^{b}	11.41	<0.001
T-SOD, U/mg pro	187.41^{a}	171.56^{b}	207.28^{a}	199.69^{a}	5.77	<0.001
MDA, nmol/mg pro	1.24^{b}	1.61^{a}	1.51^{ab}	1.38^{b}	0.09	0.036
CAT, U/mg pro	14.95	12.93	11.10	13.23	1.26	0.192
Liver						
GSH-PX, U/mg pro	158.06^{b}	158.24^{b}	174.23^{a}	167.84^{ab}	4.79	0.049
T-SOD, U/mg pro	421.54^{ab}	392.73^{b}	437.59^{a}	397.42^{b}	12.29	0.025
MDA, nmol/mg pro	0.95^{a}	1.17^{a}	1.11^{a}	0.53^{b}	0.13	0.001
CAT, U/mg pro	56.80	59.01	53.58	56.17	2.98	0.657

[1]Values are means of six replicates (2 samples per replicate).
[ab]Within a row, values with different letter superscripts mean significant difference ($P < 0.05$).
AC100, basal diet supplemented with 100 mg/kg anthocyanin + LPS challenge; AC400, basal diet supplemented with 400 mg/kg anthocyanin + LPS challenge; CAT, catalase; CON, basal diet + saline injection; GSH-Px, glutathione peroxidase; LPS, basal diet + LPS challenge; MDA, malondialdehyde; T-SOD, total superoxide dismutase.

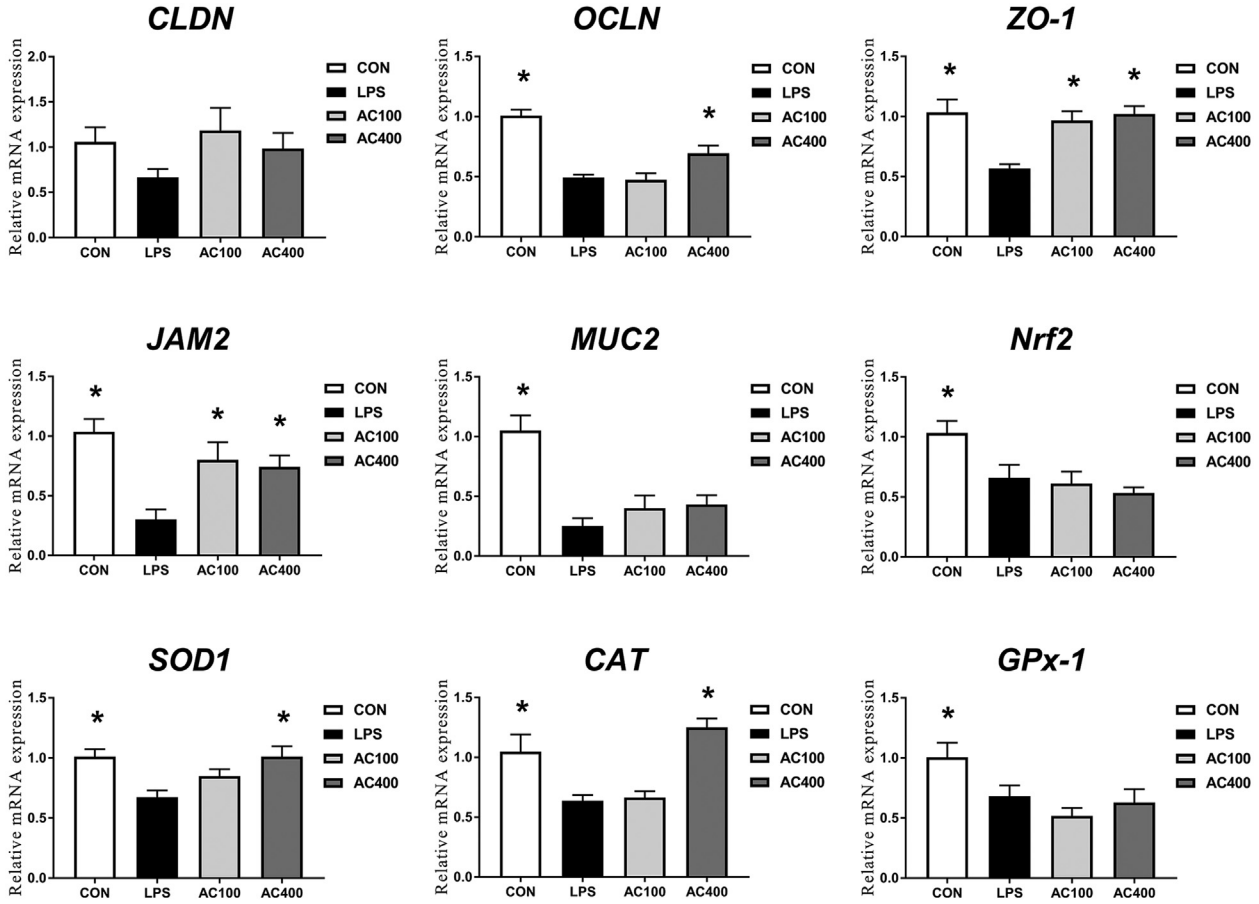

Figure 2. The relative mRNA expression of genes in jejunum mucosa. AC100, basal diet supplemented with 100 mg/kg anthocyanin + LPS challenge; AC400, basal diet supplemented with 400 mg/kg anthocyanin + LPS challenge; *CAT* = catalase; *CLDN1*, claudin 1; CON, basal diet + saline injection; *GPx-1* = cytosolic glutathione peroxidase-1; LPS, basal diet + LPS challenge; *OCLN*, occludin; *JAM*, junctional adhesion protein; *Nrf-2*, nuclear factor, erythroid 2 like 2; *NOX*, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase; *SOD1*, superoxide dismutase 1; *ZO1*, tight junction protein 1. Values are means ± SEM. $^*P < 0.05$ compared with the LPS group.

supplementation with AC could offset growth suppression of chickens after pathogen challenge, which might be associated with its anti-inflammatory and anti-oxidant activities (Zheng et al., 2016; Han et al., 2020; Zhang et al., 2021a,b) instead of direct promotion on growth performance.

Effects of AC Supplementation on Immune Function of LPS-Challenged Broiler Chicks

Liver, thymus, spleen and bursa of Fabricius are immune organs, the relative weights of which are important for immune function of poultry. Several studies showed an increase in relative weight of spleen when broiler chickens were LPS-challenged (Wang et al., 2016; Zheng et al., 2016). As the initiating event for antibody production when stimulated by antigen, LPS-induced systemic inflammation recruited inflammatory cells to the spleen, which might result in mass growth of the spleen in an acute immune response (Lin et al., 2007; Zheng et al., 2016; Yang et al., 2019). There were also different results in previous studies, for example, Shang et al (2015) found that LPS stress had no effect on relative lymphoid organ weight. Similarly, in the current research, there were no difference in relative weights of liver, spleen, thymus or bursa of Fabricius when chicks were challenged by LPS. Perhaps the LPS-induced inflammation here was insufficient to cause organ swelling.

Natural antibodies, produced by B-type lymphocytes, are essential components of the innate immune system. Liu et al. (2019) found that the LPS-enhanced intestinal immune responses included an increase in sIgA. Increased immunoglobulins could promote intestinal humoral and cellular immunity, thereby assisting in early recognition and clearance of invading pathogens in the body (Shang et al., 2015). There were different results, which showed that LPS injection decreased the IgY concentration in serum of Ross broilers (Shang et al., 2015) and sIgA in intestine of 21-d or 42-d Cobb broilers (Yang et al., 2011). In the current research, challenge with LPS decreased the IgY content in jejunal mucosa, and supplementation with 400 mg/kg AC increased the IgY content of LPS-challenged broiler chicks. The concentration of antibodies fluctuates upon receiving antigens so that there may be a transient effect beyond the current observation after LPS injection (Shang et al., 2015) (Munyaka et al., 2012). In general, the current findings suggested that AC supplementation

influenced intestinal immunity of LPS-challenged yellow-feathered broiler chicks by restoring the immunoglobulin concentration to that of the control birds.

Effects of AC Supplementation on Intestinal Morphology and Barrier of LPS-Challenged Broiler Chicks

The intestinal barrier maintains intestinal homeostasis and prevents the invasion of pathogens, and the intestinal morphology reflects the health and digestive ability of the intestine (Zheng et al., 2020). The present findings in yellow-feathered broiler chicks were consistent with previous findings that LPS challenge decreased villus height and villus height/crypt depth ratio in the small intestine (Jiang et al., 2019; Sun et al., 2020; Zheng et al., 2020). These suggested that LPS increased the permeability of the intestinal epithelial mucosa, with dysfunction of the intestine. Dietary supplementation with AC prevented the impaired jejunal integrity in LPS-challenged birds. Similarly, Csernus et al (2020) showed that AC supplementation was beneficial for the length of villi in the terminal ileum in LPS-challenged broiler chicks.

Genes of tight junctions (**TJs**), including occludin (***OCLN***), claudin (***CLDN***), junctional adhesion protein (***JAM***), and tight junction protein (***ZO-1***), play an essential part in regulating the intestinal permeability and maintaining the intestinal barrier (Gadde et al., 2017; Csernus et al., 2020). Mucin-2 (***MUC2***), produced by goblet cells, is fundamental in maintaining gel layer architecture on the luminal surface to resist pathogens (Forder et al., 2012; Lee et al., 2017; Reisinger et al., 2020). In the current research, challenge with LPS decreased the relative expression of *OCLN, ZO-1, JAM2*, and *MUC2* in jejunal mucosa of yellow-feathered broilers, consistent with the findings in Ross broilers of Lee et al (2017), Jiang et al (2019), and Gadde et al (2017). Supplementation with 100 or 400 mg/kg AC restored the relative expression of *ZO-1* and *JAM2* in LPS-injected broilers, which would mediate the improvement of epithelial barrier morphology and function. Natural plant extracts with anti-oxidant activity alleviated the damage of intestinal morphology in LPS-challenged broilers (Zhang et al., 2013; Shang et al., 2015), and the above results suggested that AC might serve as a potentially effective feed additive to protect intestinal health in young broilers.

Effects of AC Supplementation on Antioxidant Capacity of LPS-Challenged Broiler Chicks

The LPS challenge generates elevated levels of ROS, destroying the balance between pro-oxidant and antioxidant systems, thus inducing oxidative stress, which is a critical factor for the damage of the intestinal barrier (Banan et al., 2000; Zheng et al., 2016). Previous studies in broilers established that LPS challenge increased MDA content in liver (Yang et al., 2019; Zhang et al., 2021a,b) and jejunal mucosa (Jiang et al., 2019), decreased the activities of CAT and GSH-Px in jejunum and down-regulated the expression of *CAT, SOD* and *GPX1* in the jejunum (Jiang et al., 2019), which might be involved in the nuclear factor erythroid 2-related factor 2 (**Nrf2**) cascade (Zheng et al., 2016; Han et al., 2020). In the present research with young yellow-feathered broilers, LPS-induced increase in the content of MDA and decreases in activity of T-SOD and gene expression of *SOD1, CAT* and *GPX* in jejunal mucosa, were attenuated by dietary AC supplementation.

The antioxidant activity of AC has been widely established by in vitro study. For example, Bellocco et al (2016) suggested that AC in ripe pistachio showed strong antioxidant activity in oxygen radical absorbance capacity (**ORAC**), superoxide anion scavenging ($\mathbf{O^{2-}}$), 2,2-diphenyl-1-picrylhydrazyl (**DPPH**) free radical scavenging and ferric reducing antioxidant power (**FRAP**), and was clearly superior to synthetic antioxidants. Chen et al. (2019) found that raspberry extract (rich in AC) reduced the ROS levels, increased the content of GSH and activity of CAT, and ameliorated H_2O_2-induced oxidative stress in HepG2 cells through the Keap1/Nrf2 pathway. Petruk et al. (2017) found that AC from açai fruit (*Euterpe oleracea Mart.*) was able to strongly protect immortalized fibroblasts from UV-A-induced oxidative stress by inhibiting ROS production, GSH depletion, lipid peroxidation and phosphorylation of proteins involved in the oxidative stress pathway. The current study, prompted by the relative lack of information about AC on alleviation of oxidative stress in livestock and poultry, suggested that dietary supplementation with AC attenuated LPS-induced oxidative damage in yellow-feathered broilers. It is worth noting that, despite studies showing that AC activated Keap1/Nrf2 (Chen et al., 2019) or Nrf2-ARE (Kuo et al., 2019) signaling pathways to moderate anti-oxidation, such was not apparent in the current study; down-regulated *Nrf2* expression in jejunum induced by LPS was not alleviated by the supplementation with AC. The mechanism of antioxidant effect of AC in yellow-feathered broilers needs additional deeper exploration.

CONCLUSION

Dietary supplementation with AC alleviated LPS-induced reduced growth performance and intestinal mucosal damage of yellow-feathered broiler chicks by increasing gene expression of tight junction protein and improving the antioxidant status of intestinal mucosa. Based on the current findings, 100 mg/kg dietary AC is recommended to prevent intestinal oxidative injury of yellow-feathered broiler chicks. This research provides a basis for application of new feed additive products in broiler production.

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DISCLOSURES

The authors declare no conflicts of interest.

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